Characterization of macromolecular interactions by NMR and Protein Structure Determination

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Suggested texts

- NMR of macromolecules – G. C. K. Roberts
- Protein NMR Spectroscopy: Principle and practice – John Cavanagh, Wayne J. Fairbrother, Arthur G. Palmer III and Nicholas J. Skelton
- NMR of Proteins and Nucleic Acids – Kurt Wüthrich
- Biomolecular NMR Spectroscopy – Jeremy N. S. Evans
- NMR of Proteins – edited by G. M. Clore and A. M. Gronenborn
    - Spin Dynamics – Malcolm H. Levitt
    - Modern NMR techniques for chemistry research – Andrew Derome

Lecture Outline

- Introduction
- Principles of NMR: a classical, vector model
- NMR-based methods to monitor protein-ligand interactions
- Effects of chemical exchange on NMR spectra
- NMR time scale
- ^{1}H, ^{15}N HSQC titration experiments
- Mapping the binding interface
- Resonance assignment strategies
- Determination of the secondary structure
- NMR-derived structural restraints (NOEs, dihedral angles)
- Tertiary structure calculation

NMR applications and limitations

☺

- in solution - native state
- evidence of structure
- dynamics
- kinetics
- folding
- intermolecular interactions
- analytical validation of compounds
- detection of impurities

☹

- time consuming
- complex
- low sensitivity (requires mgs of a protein)
- solubility (mM concentrations)
- size limit ~ 30 kDa
- isotope labeling (^{13}C, ^{15}N, ^{2}H) is required for large proteins but can be difficult and expensive

Principles of NMR spectroscopy

- atomic nucleus has intrinsic physical properties: **mass, electric charge, magnetism and spin**
- Magnetism means that the nucleus interacts with magnetic fields like a bar magnet; spin indicates that the nucleus is "spinning around its own axis, rotating like the Earth"
- Nucleus is specified by three numbers: atomic number (number of protons), the mass number (protons+neutrons) and spin quantum number (I). Isotopes with even — Kl numbers of both protons and neutrons have I=0 (^{12}C, ^{16}O), hence cannot be characterized by NMR
- The nucleus of ^1H, a main isotope of hydrogen, contains a single proton and has spin I=1/2. ^{13}C, ^{15}N, ^{31}P –all have I=1/2 and therefore can absorb and emit energy; **emission** is monitored in NMR experiments
- Nuclei with I=1/2 possess angular momentum (as any spinning particle) and magnetic moment, μ, which arises since the nucleus has charge and spin
- The total number of energy/spin states (levels) is = $\underline{2I+1}$

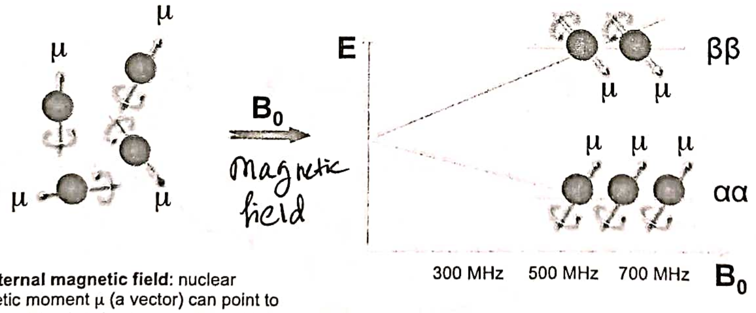
Table 1.1 Some properties of the most important elementary particles.

Particle	Rest mass (kg)	Charge	Spin
Electron	9.109×10^{-30}	$-e$	1/2
Neutron	1.675×10^{-26}	0	1/2
Proton	1.673×10^{-26}	$+e$	1/2
Photon	0	0	1

Spin dynamics, Levitt

$2I+1$ ⇒ 2 states w/ 1/2 spin #
3 states w/ 1 spin
(ground 0 + excited)

Principles of NMR: a classical, vector model



excited States

ground

No external magnetic field: nuclear magnetic moment μ (a vector) can point to "any possible direction in space" (Beware that this picture does not represent reality- the states/directions are quantised.

The important **consequence- net magnetization**, the sum of μ, $\sum\mu$=M, $\vec{M}$ **=0**

external magnetic field B_0:
Two spin states of nuclei with I=1/2:
parallel (or ground, αα) and antiparallel (excited, ββ) orientation of the z-component of μ relative to the external field B_0, **M ≠0**

Boltzman distribution ⇒ tiny bit ⇒ will more nuclei ~~[illegible]~~ in ground state

distance b/w state (energy) depends on strength of B0 300 MHz 500 MHz ie 600...

Principles of NMR: a classical, vector model

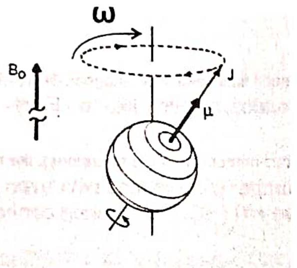


Figure 4.1 A spinning charge with angular momentum **J**, such as a proton, gives rise to a magnetic moment **μ** (= γ**J**). In a magnetic field its axis of rotation precesses around the direction of the field, like a gyroscope.

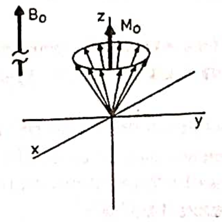


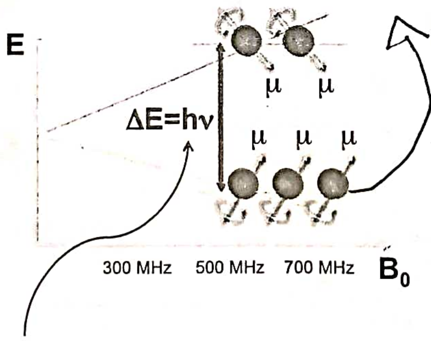
Figure 4.2 Out of a large collection of moments, a surplus have their *z* components aligned with the applied field, so the sample becomes magnetised in that direction.

Modern NMR techniques for chem res, Derome

The magnetic moment, μ, rotates around the applied external magnetic field B_0 (Z axis) at some speed, referred to as **Larmor frequency** of the nucleus, or simply NMR **absorption frequency**, ω, $\omega = -\gamma B_0$ (ω depends on the strength of the applied field)

Slightly Magnetized in parallel direction to field

Principles of NMR: a classical, vector model

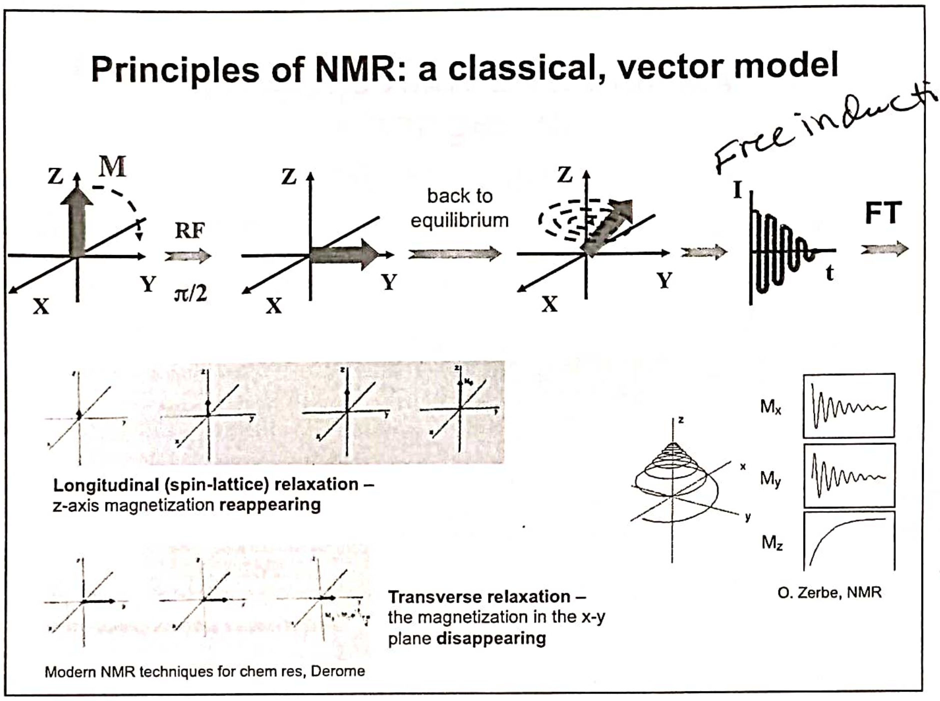


extra nuclei jump to ~~exit.~~ state

which ~~state~~ system absorb Energy and return to ground state by emitting energy

Second, RF field is applied

radio freq. low enenergy field applied -> always perpendicular to B_0 -> new energy = Gap betw states



Principles of NMR: a classical, vector model

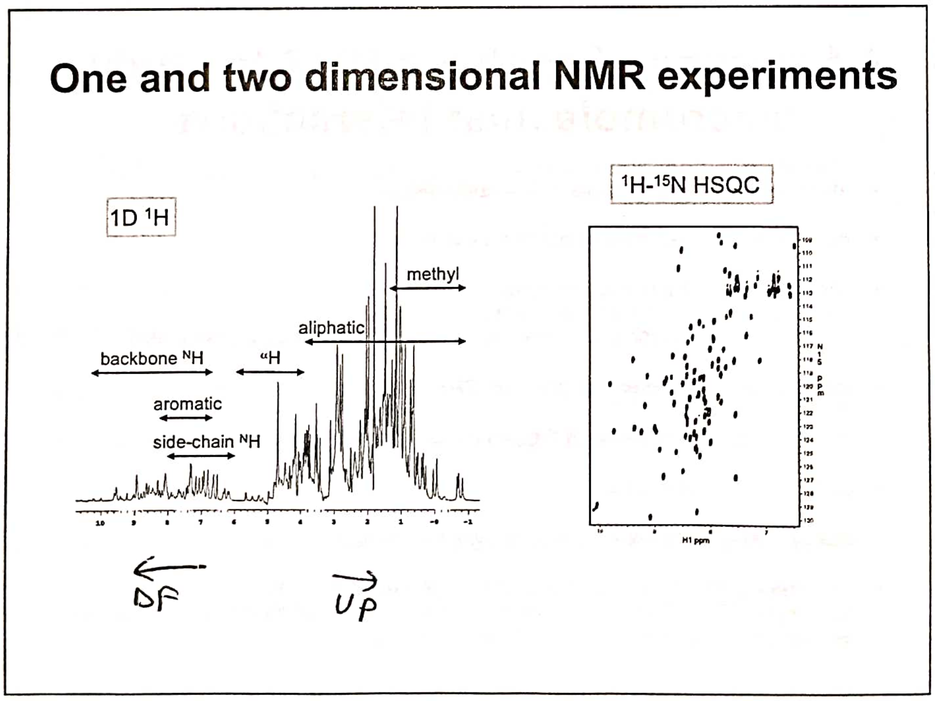
Free induction decay
FID

Longitudinal (spin-lattice) relaxation – z-axis magnetization **reappearing**

Transverse relaxation – the magnetization in the x-y plane **disappearing**

Modern NMR techniques for chem res, Derome

O. Zerbe, NMR



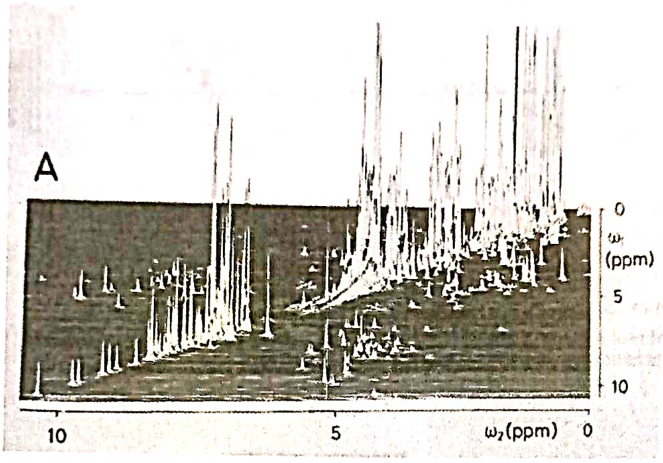
One and two dimensional NMR experiments

← DF

→ UP

Aroma ring → Planar → Shift is DF → Also the case in electro-
→ Non " → " " UP

An actual 2D NMR spectrum



NMR of Proteins and Nucleic Acids, Wüthrich

Advantage of applying NMR to study macromolecular interactions

- atomic-resolution structures of the complexes
- qualitative and quantitative binding assays
- changes in NMR parameters allow
    - to define (map) the binding sites
    - to estimate binding affinities (especially useful for weak interactions)
- rapid screening of lead compounds (SHAPES and other libraries)
- dynamic regions: motions of the protein backbone and of the side chains
- conformational changes
- characterization of multi-domain proteins in solution
- new developments: cryogenic probes, high field magnets, residual dipolar couplings, TROSY, micelles, bicelles, other mimetics of membrane, automatic resonance assignments and NOE peak picking

Interactions are monitored through changes in NMR parameters

Chemical shift: δ (corresponds to the resonance frequency of a nucleus; is related to the chemical environment of the nucleus; affected by aromatic ring currents, solvent exchange and electronegativity of nearby nuclei; units: (ppm) parts per million)

Spin-spin coupling constant: J (characterizes through-bond (scalar) interactions; related to the bond angles; causes NMR lines of coupled nuclei to split; units: Hz)

Nuclear Overhauser effect: NOE (caused by through-space (dipolar) interactions between nuclei separated in space by less than 5 A; detected by changes in NMR signal intensity of one nucleus when another is irradiated; correlated by the inverse sixth power of the distance between the nuclei)

Relaxation parameters: linewidth, T_1 and T_2 (longitudinal and transverse relaxation times)

NMR experiments

COSY: correlation spectroscopy, provides through bond connections (correlation, connectivity, interactions) for protons separated by 2 or 3 bonds

TOCSY: total correlation spectroscopy, provides through bond connections for all protons in an amino acid side chain

NOESY: nuclear Overhauser effect spectroscopy, provides through space connections between protons that are close in space

HSQC: heteronuclear single quantum coherence, provides connections between a proton and a directly attached ^{15}N or ^{13}C nucleus

Effects of chemical exchange on NMR spectra and the NMR time scale

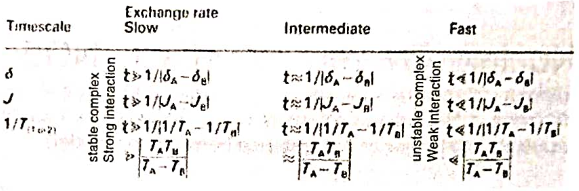
90° pulses ~ 10-20 μs

Chemical exchange is a process where a nucleus exchanges reversibly between two or more different environments, in which its NMR parameters (chemical shift (δ), spin coupling (J), or T_1 and T_2 (longitudinal and transverse relaxation times)) differ

The effect of the exchange process on the NMR spectrum depends on its rate, *k* (often k_{off}), or lifetime t=1/k relative to the magnitude of the accompanying change in the corresponding NMR parameter:

$$P + L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} PL$$

$$K_D = \frac{k_{off}}{k_{on}} = \frac{[P][L]}{[PL]}$$



Timescale	Exchange rate Slow	Intermediate	Fast
	stable complex Strong interaction		unstable complex Weak interaction
δ	$t \gg 1/\lvert\delta_A - \delta_B\rvert$	$t \approx 1/\lvert\delta_A - \delta_B\rvert$	$t \ll 1/\lvert\delta_A - \delta_B\rvert$
J	$t \gg 1/\lvert J_A - J_B\rvert$	$t \approx 1/\lvert J_A - J_B\rvert$	$t \ll 1/\lvert J_A - J_B\rvert$
$1/T_{(1\ or\ 2)}$	$t \gg 1/\lvert 1/T_A - 1/T_B\rvert$ $\gg \left\lvert\frac{T_A T_B}{T_A - T_B}\right\rvert$	$t \approx 1/\lvert 1/T_A - 1/T_B\rvert$ $\approx \left\lvert\frac{T_A T_B}{T_A - T_B}\right\rvert$	$t \ll 1/\lvert 1/T_A - 1/T_B\rvert$ $\ll \left\lvert\frac{T_A T_B}{T_A - T_B}\right\rvert$

Biomolecular NMR Spectroscopy, Evans

K_{on} and K_{off} - association and dissociation rate constants; for diffusion-controlled interactions (each collision of two molecules leads to binding) K_{on}= 10^6-10^8 1/M•sec; K_D is the equilibrium dissociation constant

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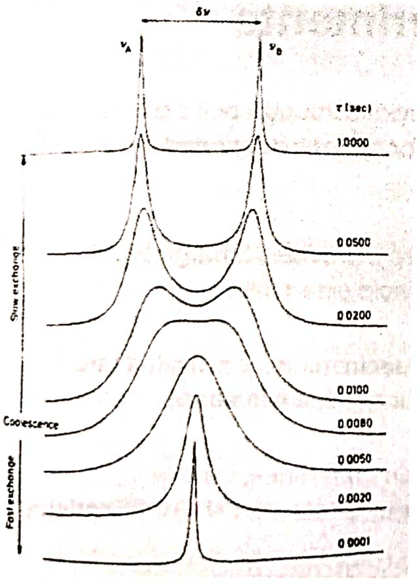
Biomolecular NMR Spectroscopy, Evans



$^a|\delta_A - \delta_B|$ is typically in the order of hundreds of hertz
$^b J$ is typically in the order of 1-10 Hz
$^c \frac{1}{T_{2A}} - \frac{1}{T_{2B}}$ is typically in the order of 1-20 Hz for protons;

Chemical exchange

Whether a system is in the fast, intermediate or slow exchange range (regime) with respect to chemical shift can be judged from the appearance of the spectrum:

Fast- a single resonance line is observed with a chemical shift being a weighted average of chem shifts of the individual species

Intermediate- resonances broaden markedly, often becoming unobservable

Slow- separate resonances are seen for the nucleus in each of the two states

600 = FAST →
900 = Slow

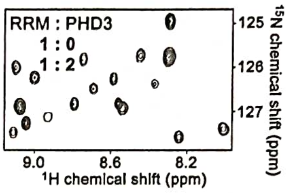
Examples of the fast, intermediate and slow exchange regimes on the NMR time scale

The exchange regime depends on k_{off}, (the stronger the interaction- the more it's shifted toward the slow exchange) and on the chemical shift difference (in Hz) between the two states.

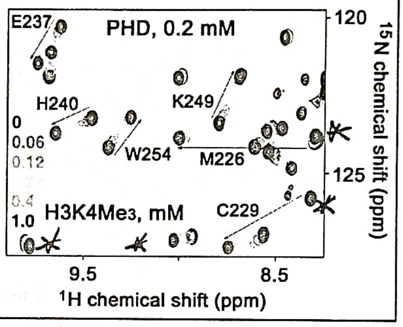
Consequence: different subsets of peaks can be in different exchange regimes in the same titration experiment. For example, peaks that move farther, could be in intermediate exchange, whereas peaks that move less would be in fast exchange regime.

Because the chem shift difference is expressed in Hz, its value is proportional to B_0. The use of a higher-field spectrometer shifts the regime toward slow exchange.

slow

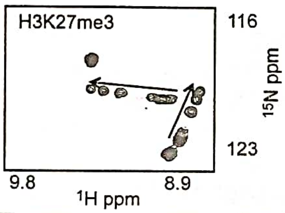


Intermediate-to-fast

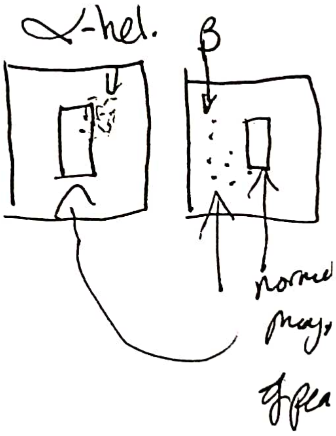


→ different sizes broaden

fast

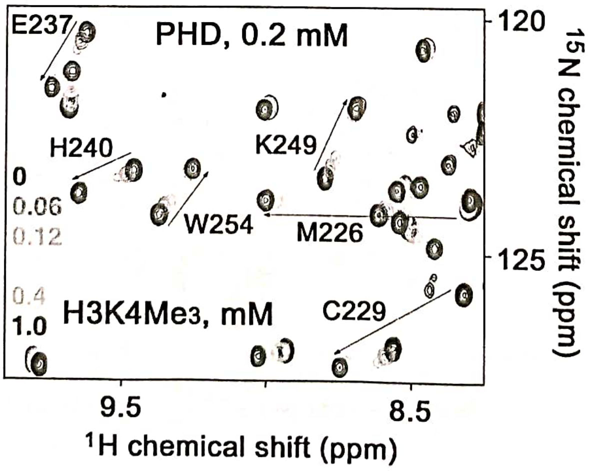


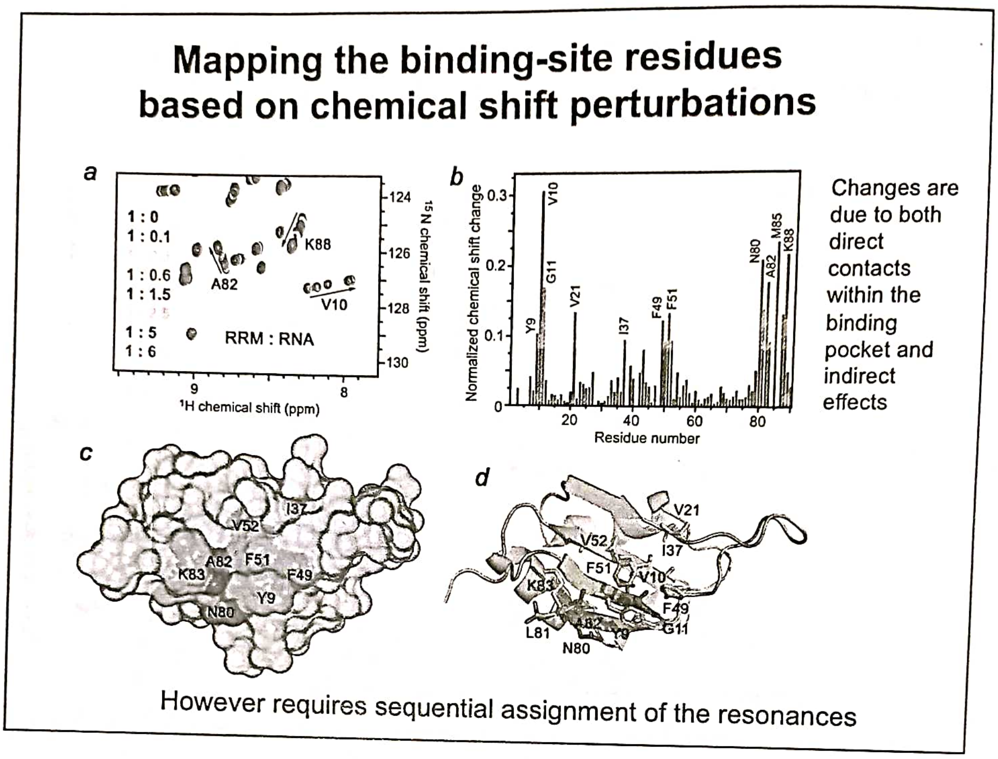
all peaks same size/intensity



^{1}H, ^{15}N HSQC titration is a common NMR experiment to study interactions

1. Testing for binding- Yes/No answer
2. No prior knowledge about the ligand (or protein) is necessary
3. Protein is folded or not
4. Contains folded and/or unfolded fragments
5. α-helical or β-strand-containing
6. Binding is weak or tight
7. Number of residues affected

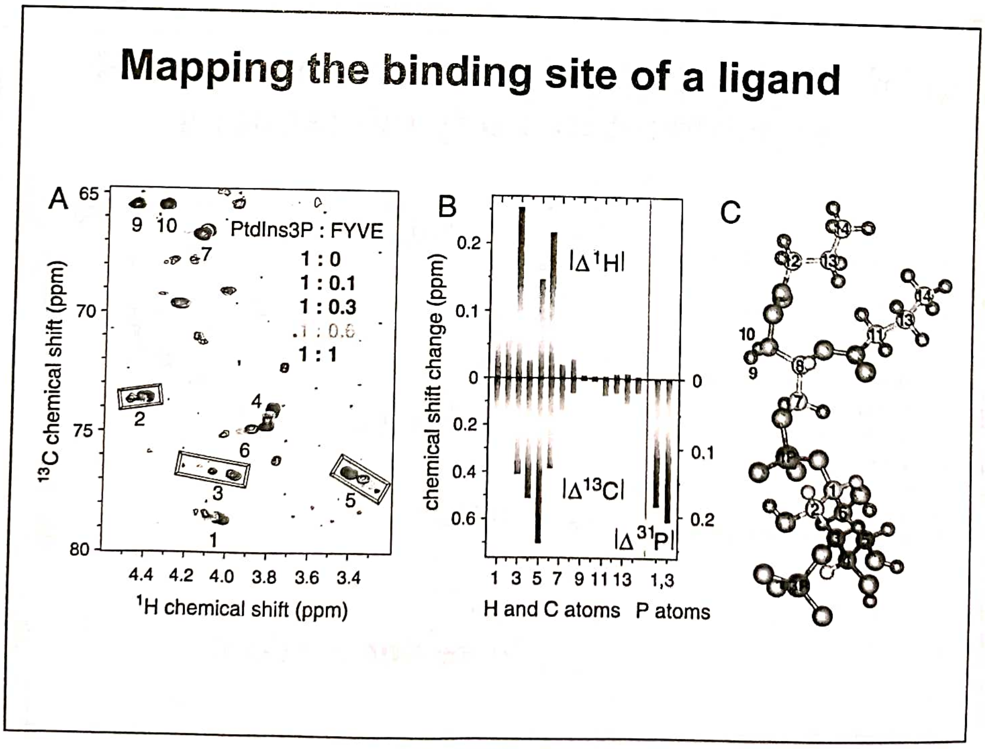




Mapping the binding-site residues based on chemical shift perturbations

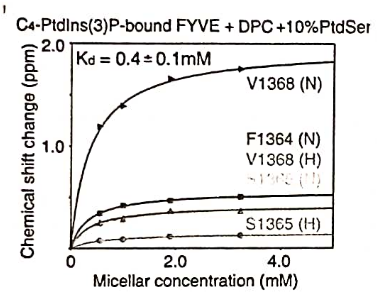
Changes are due to both direct contacts within the binding pocket and indirect effects

However requires sequential assignment of the resonances



Mapping the binding site of a ligand

K_Ds can be measured from NMR titrations

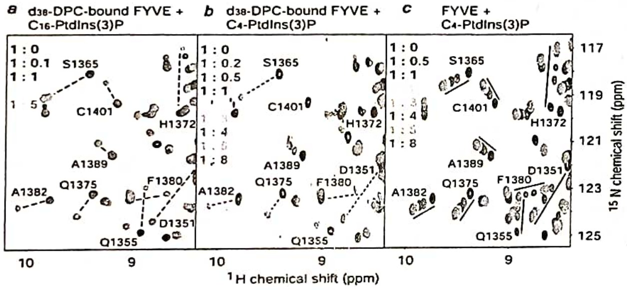


$$\Delta\delta = \Delta\delta_{max}\left(\left([L]+[P]+K_d\right)-\sqrt{\left([L]+[P]+K_d\right)^2-4[P][L]}\right)\Big/2[P]$$

Applicable when K_D is in the µM-mM range, because of high protein concentration ([P] ~100 µM)

For a strong interaction, in the pM-nM range- only an upper limit for the K_D can be estimated

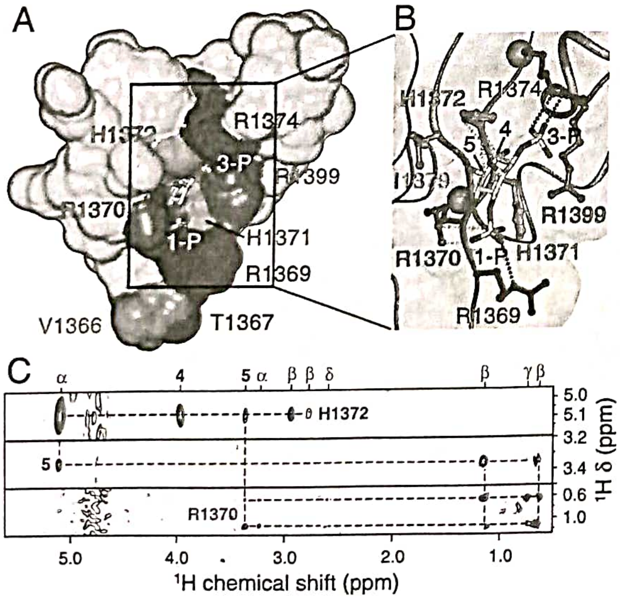
benefits: fast, low protein concentration, even very weak binding, no false positive/negative



→ Kds
↳ Kd > [P]
Kd = [P][L] / [PL]

Kd = [L] when [P] = P[L] = 50.
Not the case for NMR/ITC.
(negligable)

Intermolecular NOEs – interproton distances (shorter than 5Å)



Labeling with stable isotopes, such as ^{15}N, ^{13}C and/or ^{2}H

the use of isotope - filtered and -edited experiments allows to select only the intermolecular signals

Other NMR-based methods for the identification of direct intermolecular contacts

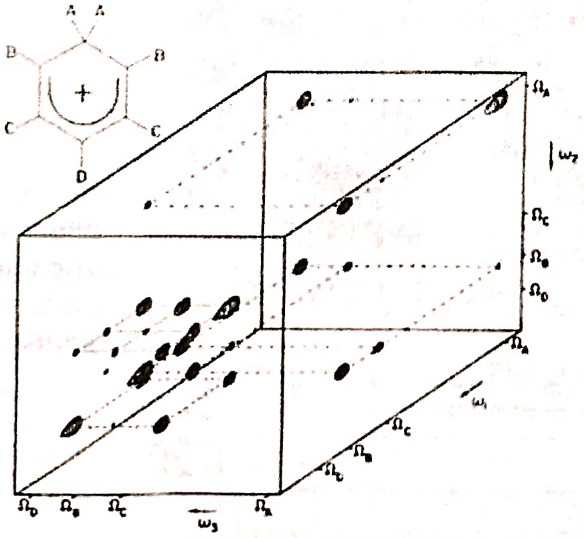
1. **Cross-saturation or saturation transfer (SAT)**: the protein is ^{2}H- and ^{15}N-labeled with its amide deuterons exchanged back to protons. The ligand is unlabeled. Saturation of the ligand (irradiation/suppression of its signals) results in attenuation of signals of those residues of the protein that are located in close proximity (cross-relaxation mechanism, i.e. saturation is transferred through space). Often monitored by ^{1}H,^{15}N HSQC.

2. **Paramagnetic labels:** A paramagnetic label, attached to a Cys located near the binding interface, attenuates NMR resonances of the interacting proteins over a longer range, up to 20Å due to electron-nuclei dipolar interactions. Provides additional intermolecular distance restraints.

3. **Mapping the binding interface with dynamics**: ^{15}N relaxation can be used to examine the dynamics of the protein backbone. Information is obtained on rapid fluctuations at the pico to nano-second time scale and on motions at the milli and microsecond time scale.. Measurements of R1 and R2 relaxation rates and heteronuclear ^{1}H-^{15}N NOEs.

4. **Residual dipolar coupling (RDC)**: Similarly to a scalar coupling (J), the dipolar coupling causes splitting of NMR signals. The splitting depends on the orientation of the N-H dipole-dipole vector with respect to the magnetic field. A weak alignment of protein molecules in an anisotropic medium (bicelles, gels) leads to a residual coupling. Is useful to calculate the alignment tensor and determine the relative orientation of two domains.

Protein structure determination using 2D, 3D and 4D spectra of ^{15}N/^{13}C labeled proteins



Protein Data Bank: www.rcsb.org/pdb/index.html
Research Collaboration for Structural Bioinformatics

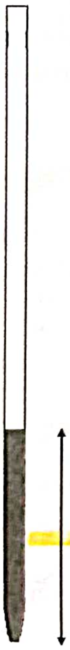
Total 88,714 (5 March 2013)
71,955 (29 March 2011)
55,419 (25 January 2009)
41,013 structures (9 January 2007)
18,359 structures (1 August 2002)
3,298 structures in 2001
~90% - crystal
~10% - solution structures

BioMagResBank: www.bmrb.wisc.edu/Welcome.html
University of Wisconsin-Madison

8,266 – proteins/peptides
221 – DNA
212 - RNA

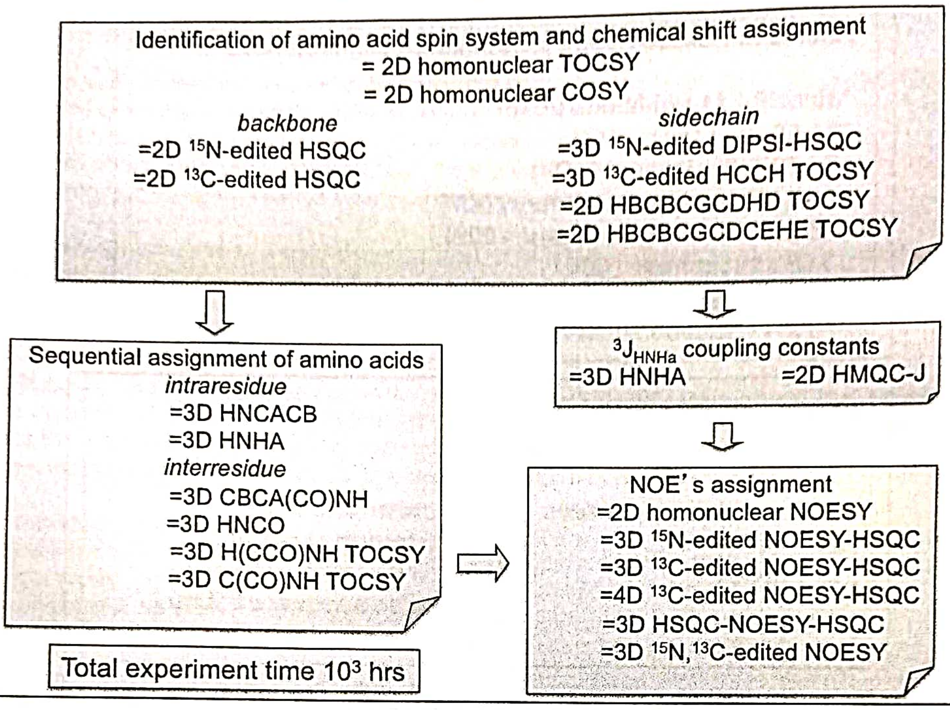
chemical shifts
coupling constants
NMR software library
pulse sequence library
reference information

NMR sample



- 550 µl of 0.1-2 mM unlabeled, ^{15}N- or ^{15}N/^{13}C- isotope labeled protein
- deuterated or phosphate buffer
- pH 2-7
- at least 95% purity
- not aggregated
- stable for several days/weeks
- d_{10}-DTT (Cys)
- NaN_3

NMR experiments



Identification of amino acid spin system and chemical shift assignment
= 2D homonuclear TOCSY
= 2D homonuclear COSY

backbone
=2D ^{15}N-edited HSQC
=2D ^{13}C-edited HSQC

sidechain
=3D ^{15}N-edited DIPSI-HSQC
=3D ^{13}C-edited HCCH TOCSY
=2D HBCBCGCDHD TOCSY
=2D HBCBCGCDCEHE TOCSY

Sequential assignment of amino acids
intraresidue
=3D HNCACB
=3D HNHA
interresidue
=3D CBCA(CO)NH
=3D HNCO
=3D H(CCO)NH TOCSY
=3D C(CO)NH TOCSY

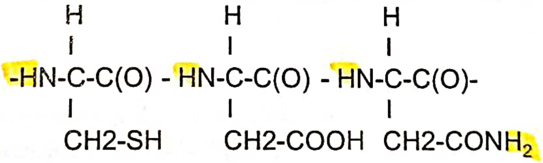
$^{3}J_{HNHa}$ coupling constants
=3D HNHA =2D HMQC-J

NOE's assignment
=2D homonuclear NOESY
=3D ^{15}N-edited NOESY-HSQC
=3D ^{13}C-edited NOESY-HSQC
=4D ^{13}C-edited NOESY-HSQC
=3D HSQC-NOESY-HSQC
=3D ^{15}N,^{13}C-edited NOESY

Total experiment time 10^3 hrs

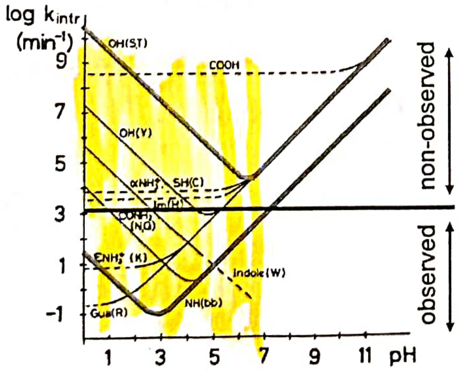
Labile protons

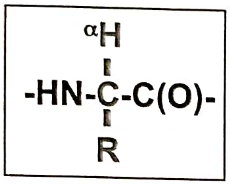
Cys-Asp-Asn



$$\begin{array}{ccc} \text{H} & \text{H} & \text{H} \\ | & | & | \\ \text{-HN-C-C(O) -} & \text{HN-C-C(O) -} & \text{HN-C-C(O)-} \\ | & | & | \\ \text{CH2-SH} & \text{CH2-COOH} & \text{CH2-CONH}_2 \end{array}$$

Labile but often NMR-observable protons
Labile protons that are not observed

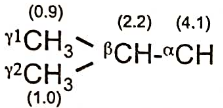
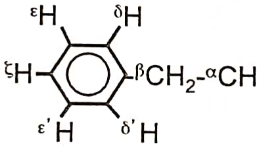




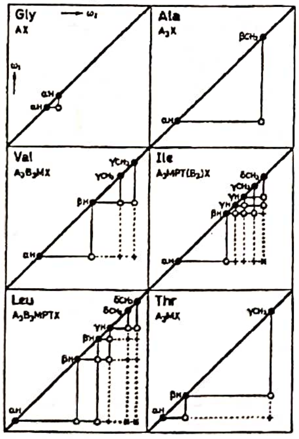
$$\begin{array}{c} ^{\alpha}H \\ | \\ -HN-C-C(O)- \\ | \\ R \end{array}$$

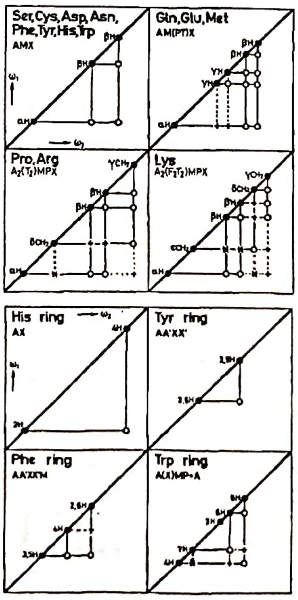
Spin systems of nonlabile protons in amino acids

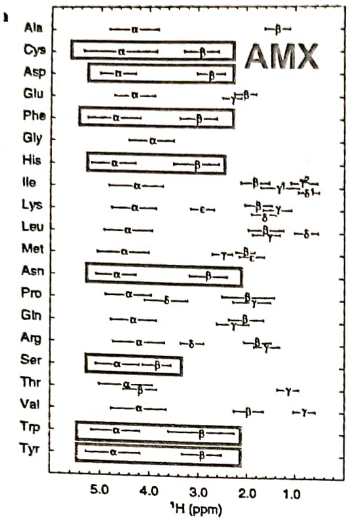
a group of spins, connected by scalar (through bond) spin-spin coupling

4.0 ppm (0.34 – 6.17) $^{\alpha}H$-$^{\alpha}CH$	Gly, G	AX (AB or A_2)
(1.3) (4.3) $^{\beta}CH_3$-$^{\alpha}CH$	Ala, A	A_3X
 (0.9) (2.2) (4.1) $^{\gamma 1}CH_3$ $^{\beta}CH$-$^{\alpha}CH$ $^{\gamma 2}CH_3$ (1.0)	Val, V	A_3B_3MX
(2.9) (1.6) (1.4) (1.8) (4.3) $\overset{+}{N}H_3$-$^{\varepsilon}CH_2$-$^{\delta}CH_2$-$^{\gamma}CH_2$-$^{\beta}CH_2$-$^{\alpha}CH$	Lys, K	$A_2(F_2T_2)MPX$
 $^{\varepsilon}H$ $^{\delta}H$ $^{\zeta}H$ $^{\beta}CH_2$-$^{\alpha}CH$ $\varepsilon'H$ $\delta'H$	Phe, F	AMX+AMM’XX’

COSY connectivity diagrams for the spin systems of nonlabile protons in the 20 common amino acids

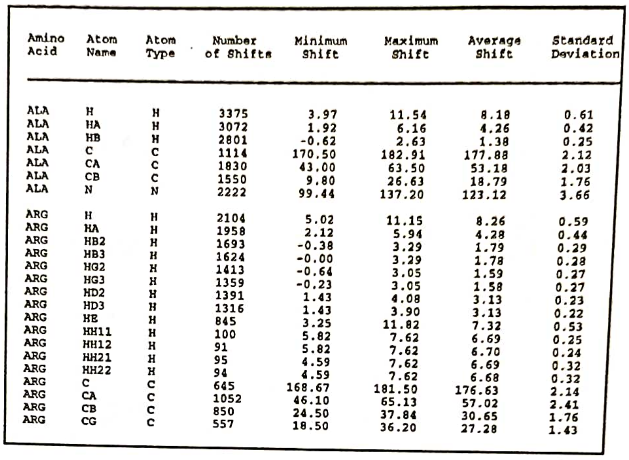






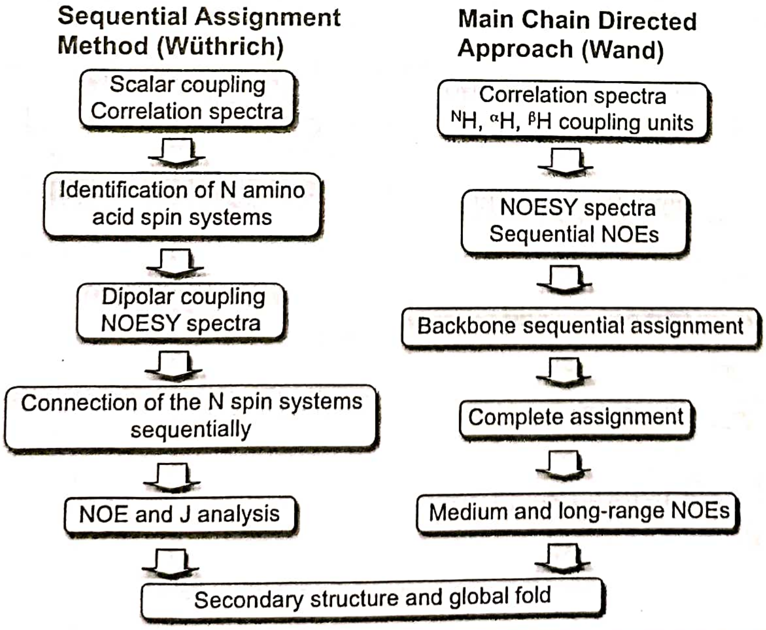
Aliphatic ^{1}H chemical shifts obtained from the database of 13 folded proteins

Statistics of the ^{1}H, ^{13}C and ^{15}N chemical shifts (BMRB database; 316,848 chemical shifts)



Amino Acid	Atom Name	Atom Type	Number of Shifts	Minimum Shift	Maximum Shift	Average Shift	Standard Deviation
ALA	H	H	3375	3.97	11.54	8.18	0.61
ALA	HA	H	3072	1.92	6.16	4.26	0.42
ALA	HB	H	2801	-0.62	2.63	1.38	0.25
ALA	C	C	1114	170.50	182.91	177.88	2.12
ALA	CA	C	1830	43.00	63.50	53.18	2.03
ALA	CB	C	1550	9.80	26.63	18.79	1.76
ALA	N	N	2222	99.44	137.20	123.12	3.66
ARG	H	H	2104	5.02	11.15	8.26	0.59
ARG	HA	H	1958	2.12	5.94	4.28	0.44
ARG	HB2	H	1693	-0.38	3.29	1.79	0.29
ARG	HB3	H	1624	-0.00	3.29	1.78	0.28
ARG	HG2	H	1413	-0.64	3.05	1.59	0.27
ARG	HG3	H	1359	-0.23	3.05	1.58	0.27
ARG	HD2	H	1391	1.43	4.08	3.13	0.23
ARG	HD3	H	1316	1.43	3.90	3.13	0.22
ARG	HE	H	845	3.25	11.82	7.32	0.53
ARG	HH11	H	100	5.82	7.62	6.69	0.25
ARG	HH12	H	91	5.82	7.62	6.70	0.24
ARG	HH21	H	95	4.59	7.62	6.69	0.32
ARG	HH22	H	94	4.59	7.62	6.68	0.32
ARG	C	C	645	168.67	181.50	176.63	2.14
ARG	CA	C	1052	46.10	65.13	57.02	2.41
ARG	CB	C	850	24.50	37.84	30.65	1.76
ARG	CG	C	557	18.50	36.20	27.28	1.43

Sequence specific resonance assignment based on homonucler ^{1}H NMR spectra only



Sequential Assignment Method (Wüthrich)

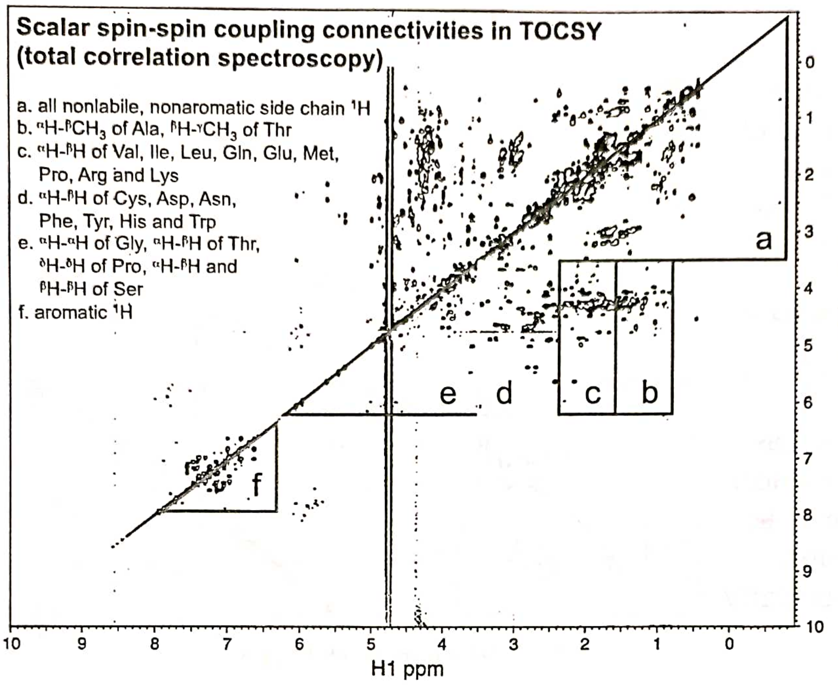
- Scalar coupling Correlation spectra
- Identification of N amino acid spin systems
- Dipolar coupling NOESY spectra
- Connection of the N spin systems sequentially
- NOE and J analysis

Main Chain Directed Approach (Wand)

- Correlation spectra ^{N}H, $^{\alpha}$H, $^{\beta}$H coupling units
- NOESY spectra Sequential NOEs
- Backbone sequential assignment
- Complete assignment
- Medium and long-range NOEs

Secondary structure and global fold

Scalar spin-spin coupling connectivities in TOCSY (total correlation spectroscopy)



a. all nonlabile, nonaromatic side chain ^1H

b. $^{\alpha}H$-$^{\beta}CH_3$ of Ala, $^{\beta}H$-$^{\gamma}CH_3$ of Thr

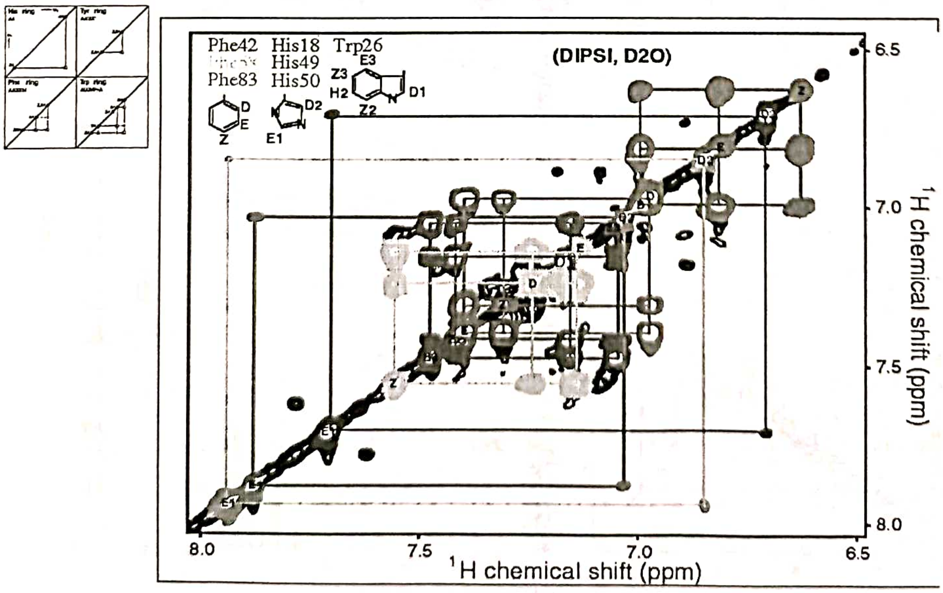
c. $^{\alpha}H$-$^{\beta}H$ of Val, Ile, Leu, Gln, Glu, Met, Pro, Arg and Lys

d. $^{\alpha}H$-$^{\beta}H$ of Cys, Asp, Asn, Phe, Tyr, His and Trp

e. $^{\alpha}H$-$^{\alpha}H$ of Gly, $^{\alpha}H$-$^{\beta}H$ of Thr, $^{\delta}H$-$^{\delta}H$ of Pro, $^{\alpha}H$-$^{\beta}H$ and $^{\beta}H$-$^{\beta}H$ of Ser

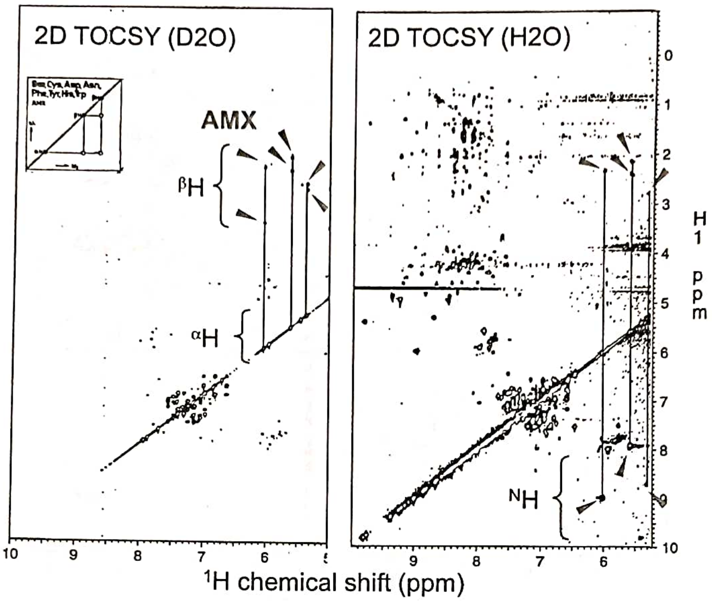
f. aromatic ^1H

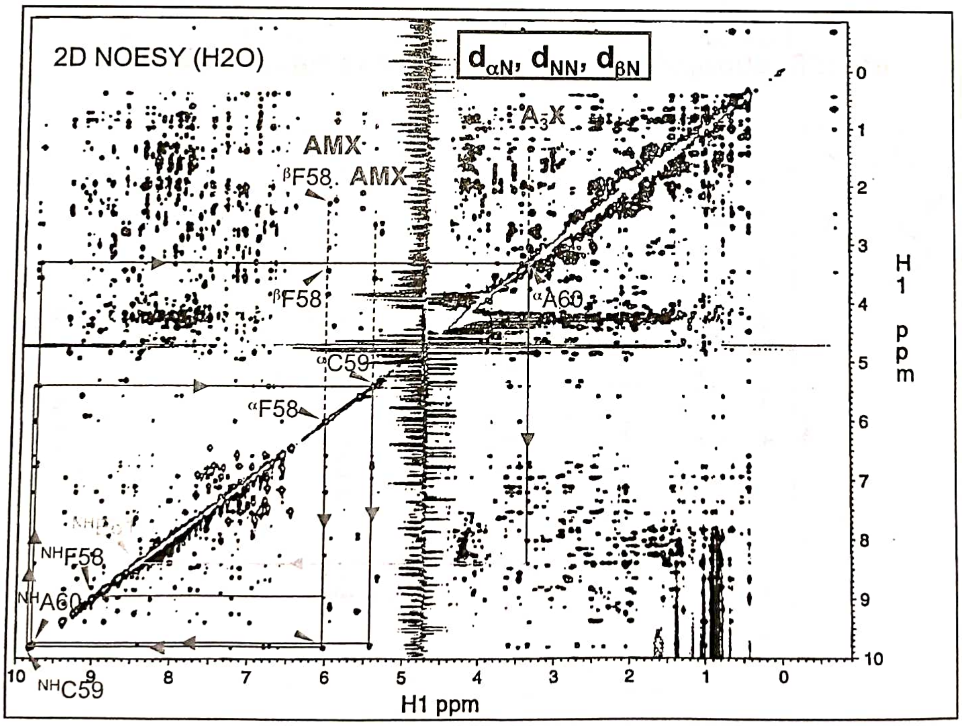
Identification of ^1H spin systems in aromatic residues

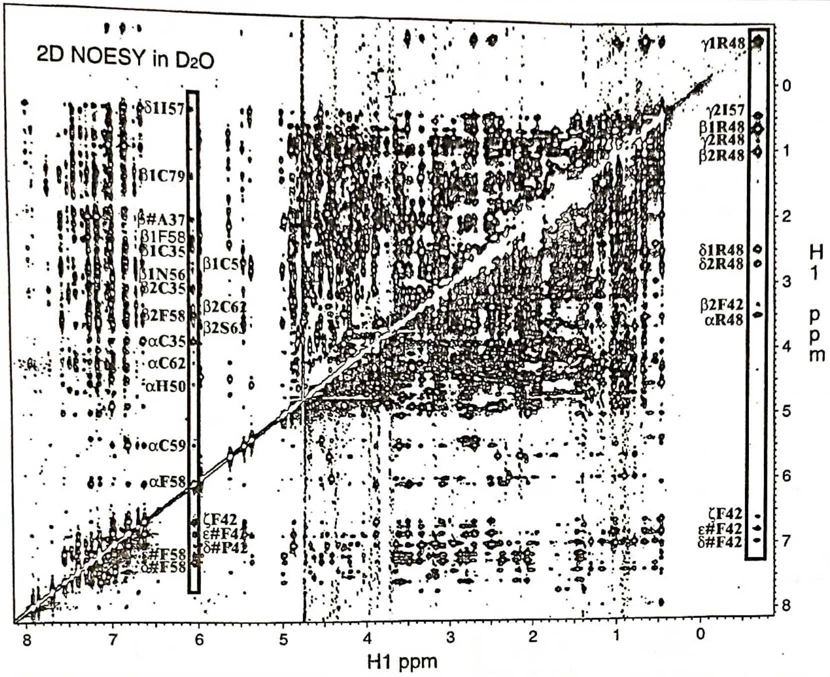


Connection of the spin systems with labile amides

Next slide: Connection of the spin systems sequentially







Resonance assignment based on heteronuclear spectra

Triple-resonance experiments correlate backbone ^NH, N, $^\alpha H$, $^\alpha C$, CO and side chain $^\beta H$ and $^\beta C$ spins using one- and two-bond scalar coupling

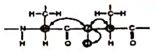
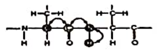
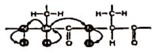
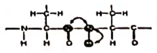
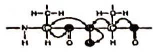
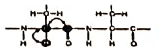
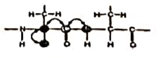
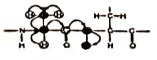
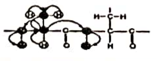
$^1H^N \rightarrow {}^{15}N \rightarrow {}^{13}CO \rightarrow$
(t_1)

$^{13}C^\alpha \rightarrow {}^{13}CO \rightarrow {}^{15}N \rightarrow {}^1H^N$
(t_2) (t_3)

⇩

HN(CO)CA

$^1J_{HN}$ ~91 Hz
$^1J_{NC\alpha}$~7-11 Hz
$^2J_{NC\alpha}$~4-9 Hz
$^1J_{NCO}$~15 Hz
$^1J_{C\alpha CO}$~55 Hz
$^1J_{CC}$~35 Hz
$^1J_{CH}$~140 Hz

Experiment	Correlations observed	Magnetization transfer	J Couplings^b
HNCA	$^1H^N_i-{}^{15}N_i-{}^{13}C^\alpha_i$ $^1H^N_i-{}^{15}N_i-{}^{13}C^\alpha_{i-1}$		$^1J_{NH}$ $^1J_{NC^\alpha}$ $^2J_{NC^\alpha}$
HN(CO)CA	$^1H^N_i-{}^{15}N_i-{}^{13}C^\alpha_{i-1}$		$^1J_{NH}$ $^1J_{NCO}$ $^1J_{C^\alpha CO}$
H(CA)NH	$^1H^\alpha_i-{}^{15}N_i-{}^1H^N_i$ $^1H^\alpha_i-{}^{15}N_{i+1}-{}^1H^N_{i+1}$		$^1J_{C^\alpha H^\alpha}$ $^1J_{NC^\alpha}$ $^2J_{NC^\alpha}$ $^1J_{NH}$
HNCO	$^1H^N_i-{}^{15}N_i-{}^{13}CO_{i-1}$		$^1J_{NH}$ $^1J_{NCO}$
HN(CA)CO	$^1H^N_i-{}^{15}N_i-{}^{13}CO_i$ $^1H^N_i-{}^{15}N_i-{}^{13}CO_{i-1}$		$^1J_{NH}$ $^1J_{NC^\alpha}$ $^2J_{NC^\alpha}$ $^1J_{C^\alpha CO}$
HCACO	$^1H^\alpha_i-{}^{13}C^\alpha_i-{}^{13}CO_i$		$^1J_{C^\alpha H^\alpha}$ $^1J_{C^\alpha CO}$
HCA(CO)N	$^1H^\alpha_i-{}^{13}C^\alpha_i-{}^{15}N_{i+1}$		$^1J_{C^\alpha H^\alpha}$ $^1J_{C^\alpha CO}$ $^1J_{NCO}$
CBCA(CO)NH	$^{13}C^\beta_i-{}^{13}C^\alpha_i-{}^{15}N_{i+1}-{}^1H^N_{i+1}$		$^1J_{CH}$ $^1J_{C^\alpha C^\beta}$ $^1J_{C^\alpha CO}$ $^1J_{NCO}$ $^1J_{NH}$
CBCANH	$^{13}C^\beta_i/{}^{13}C^\alpha_i-{}^{15}N_i-{}^1H^N_i$ $^{13}C^\beta_i/{}^{13}C^\alpha_i-{}^{15}N_{i+1}-{}^1H^N_{i+1}$		$^1J_{CH}$ $^1J_{C^\alpha C^\beta}$ $^1J_{NC^\alpha}$ $^2J_{NC^\alpha}$ $^1J_{NH}$

Protein structure determination using 3D and 4D spectra of $^{15}N/^{13}C$ labeled proteins

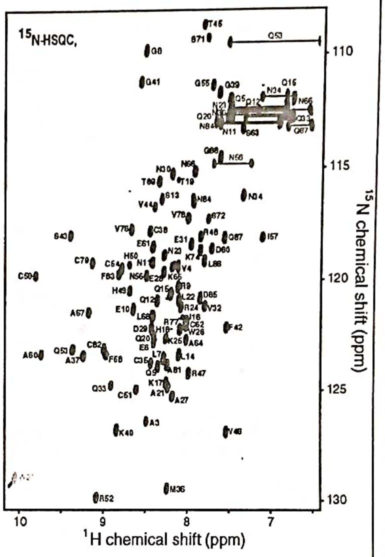
Sequential assignment of amino acids using triple-resonance *HNCACB, CBCA(CO)NH* + *^1H-^{15}N HSQC*

Identification of spin systems and complete chemical shift assignment
intraresidue: 3D ^{15}N- and ^{13}C-edited TOCSY,
Interresidue: 3D HCC and CCC TOCSY, HNCO

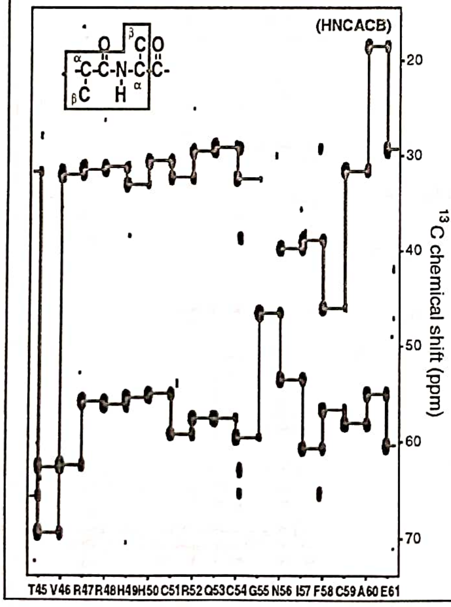
NOEs: 3D ^{15}N- and ^{13}C-edited NOESY

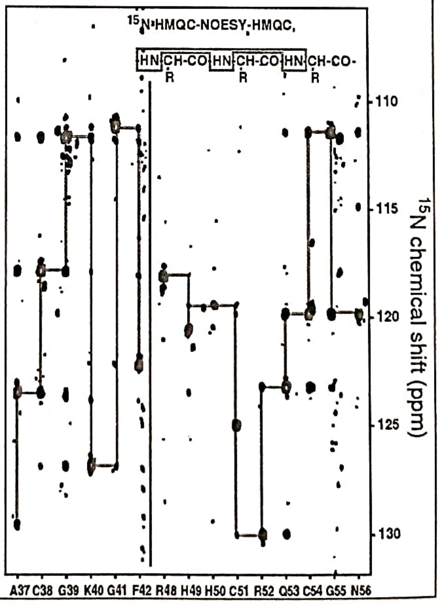
$^3J_{HNH\alpha}$ coupling constants: 3D HNHA, HMQC-J

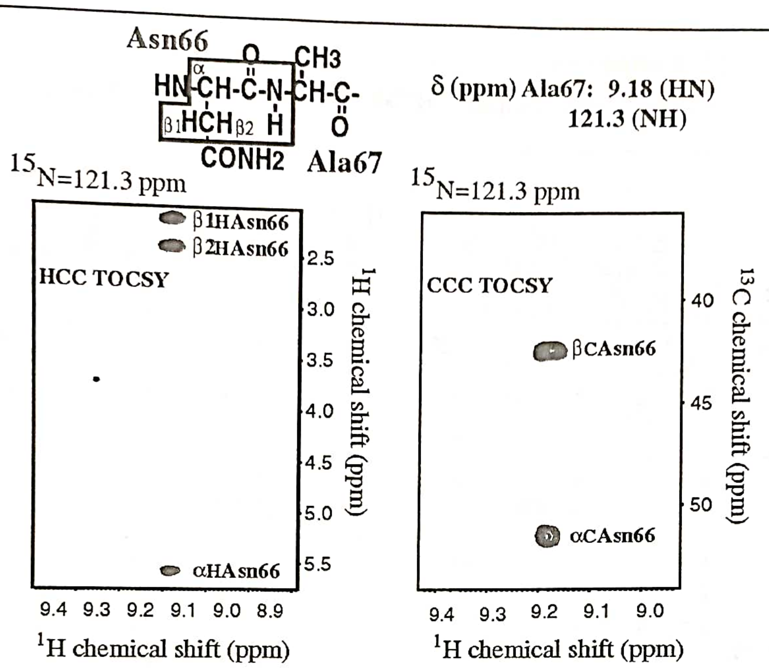
Structure calculation



Sequential resonance assignment







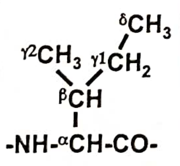
δ (ppm) Ala67: 9.18 (HN)
121.3 (NH)

Assignment of aliphatic ^1H and ^{13}C resonances using ^{13}C-^{13}C correlations. HCCH-TOCSY

^1H-^{13}C-^{13}C-^1H

$^1J_{CC}$~35 Hz

$^1J_{CH}$~140 Hz



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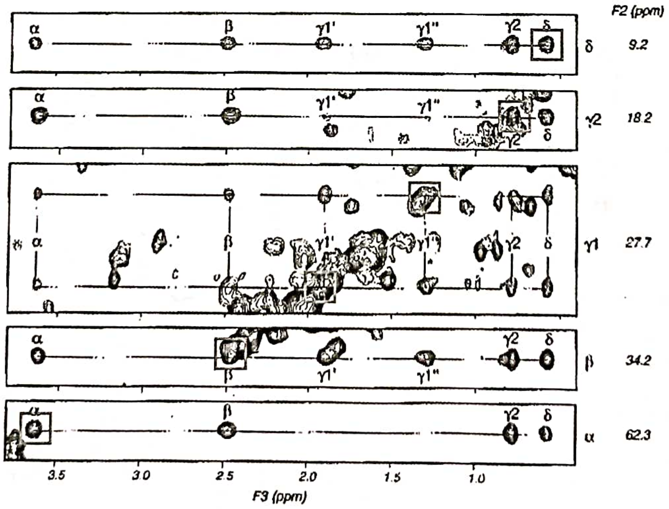
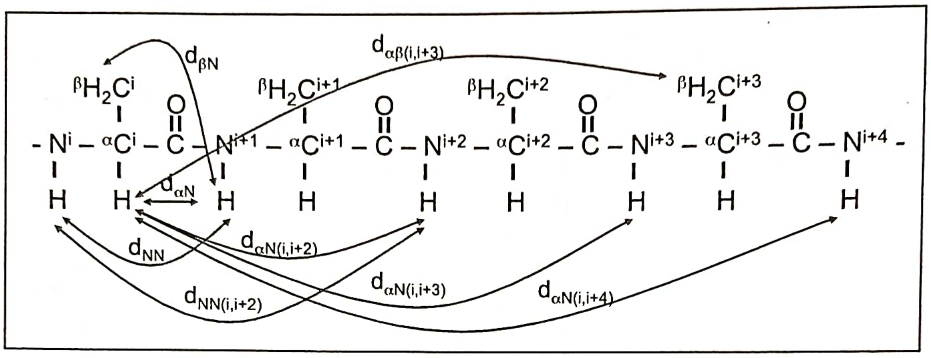


NOEs - observable ^1H-^1H distances in proteins

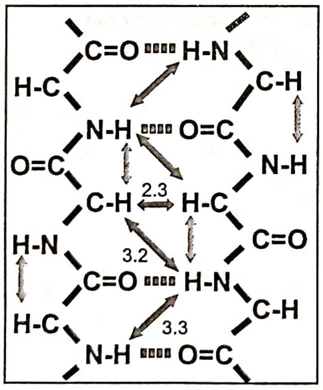


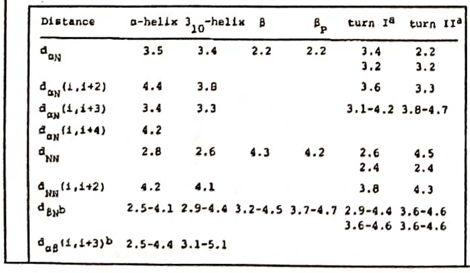
^1H-^1H distances:
- intraresidue
- sequential (short-range)
- medium-range (j-i)=2-5
- long-range (j-i)≥6

$\boxed{I_{NOE} \propto r^{-6}}$

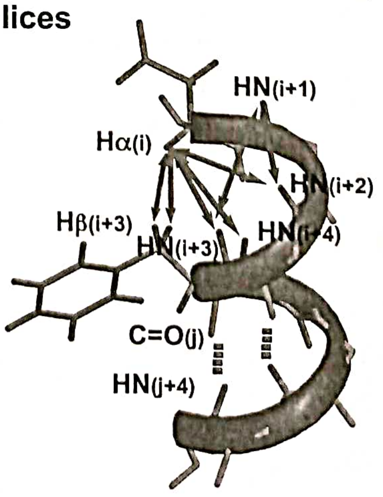
NOEs: weak
medium
strong
Limits: 2 – 5 Å

Short sequential and long-range backbone ^1H-^1H distances in antiparallel β sheets and short sequential and medium-range in α helices

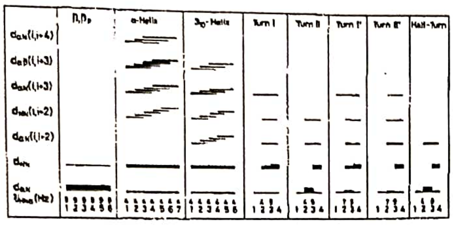


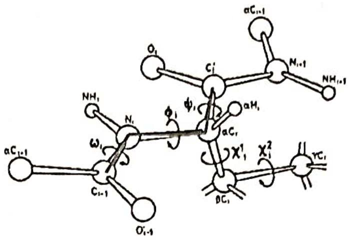


Distance	α-helix	3_{10}-helix	β	$β_P$	turn I[a]	turn II[a]
$d_{\alpha N}$	3.5	3.4	2.2	2.2	3.4 3.2	2.2 3.2
$d_{\alpha N}(i,i+2)$	4.4	3.8			3.6	3.3
$d_{\alpha N}(i,i+3)$	3.4	3.3			3.1-4.2	3.8-4.7
$d_{\alpha N}(i,i+4)$	4.2					
d_{NN}	2.8	2.6	4.3	4.2	2.6 2.4	4.5 2.4
$d_{NN}(i,i+2)$	4.2	4.1			3.8	4.3
$d_{\beta N}$[b]	2.5-4.1	2.9-4.4	3.2-4.5	3.7-4.7	2.9-4.4 3.6-4.6	3.6-4.6 3.6-4.6
$d_{\alpha\beta}(i,i+3)$[b]	2.5-4.4	3.1-5.1				



Summary of the sequential and medium-range NOEs and spin-spin coupling constants in the secondary structure elements

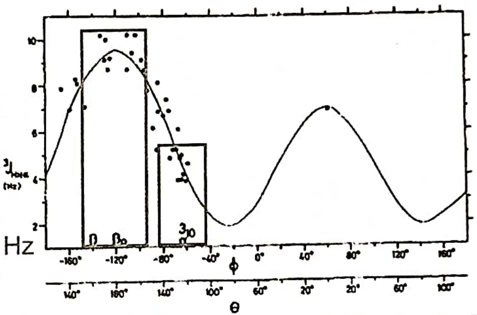


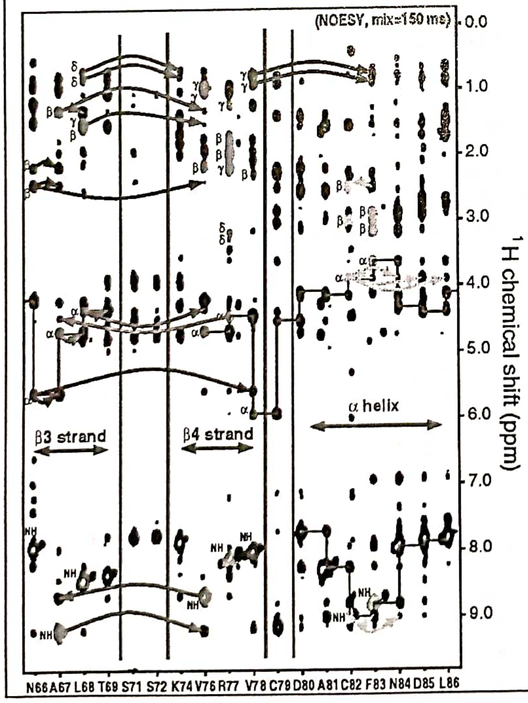


Size of $^3J_{HNH\alpha}$ depends on the dihedral angle Φ (Φ=θ - 60°)

$$^3J_{HNH\alpha}=6.4\cos^2\theta - 1.4\cos\theta + 1.9$$

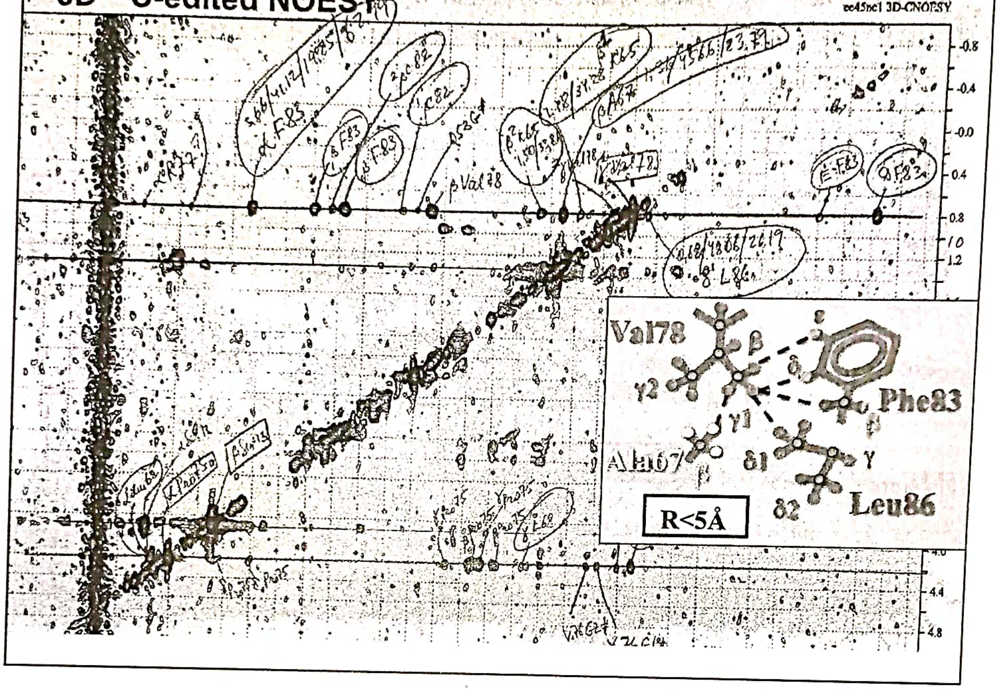
α helix (Φ = -57°), $^3J_{HNH\alpha}$= 3.9 Hz
β sheet (Φ = -120°), $^3J_{HNH\alpha}$= 8.9-9.7 Hz





Secondary structure determination and collection of the d_{NX} restraints in 3D ^{15}N-edited NOESY

3D ^{13}C-edited NOESY



Computational approach to determine the structure of a protein

Input:

1. known sequence of the amino acid residues
2. known molecular structure of the residues, including bond distances and angles
3. known planar structure of the peptide group
4. NMR-derived restraints:

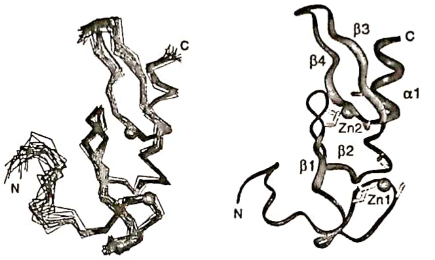
- Interproton distances (NOEs)
  weak, medium and strong intensity
- Intra- and interresidue bond angles
  Φ, Ψ, χ^n
- Hydrogen bonds
  N-O and H-O distances
- Heteroatom distance and angle restraints
  Ca^{2+}, Mg^{2+}, Zn^{2+}, etc.

Calculation of the 3 dimensional structure

- Distance geometry
- Restrained molecular dynamics simulated annealing

Input

Minimization of system energy:
bond lenght and angles,
van der Waals
Minimization of violations:
NOEs, dihedral angles
Several cycles - calculation-
analysis of violated restraints



A set of 20 lowest energy structures

Average structure

Input restraint summary

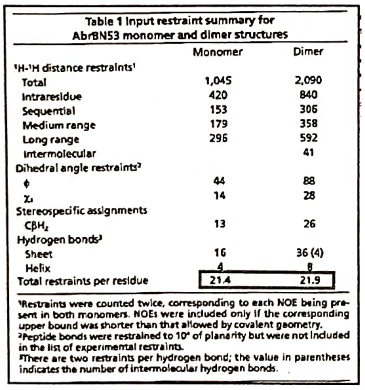


Table 1 Input restraint summary for AbrBN53 monomer and dimer structures

	Monomer	Dimer
^{1}H-^{1}H distance restraints[1]		
Total	1,045	2,090
Intraresidue	420	840
Sequential	153	306
Medium range	179	358
Long range	296	592
Intermolecular		41
Dihedral angle restraints[2]		
ϕ	44	88
χ_1	14	28
Stereospecific assignments		
CβH$_2$	13	26
Hydrogen bonds[3]		
Sheet	16	36 (4)
Helix	4	8
Total restraints per residue	21.4	21.9

[1] Restraints were counted twice, corresponding to each NOE being present in both monomers. NOEs were included only if the corresponding upper bound was shorter than that allowed by covalent geometry.
[2] Peptide bonds were restrained to 10° of planarity but were not included in the list of experimental restraints.
[3] There are two restraints per hydrogen bond; the value in parentheses indicates the number of intermolecular hydrogen bonds.

Output: Structural statistics

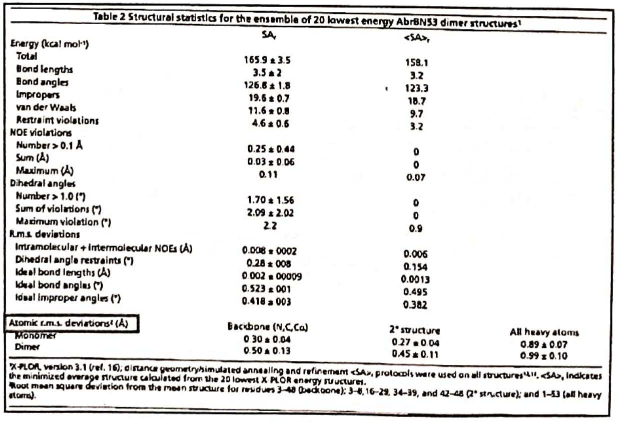
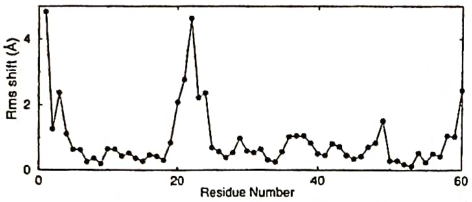


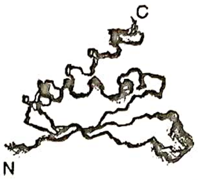
Table 2 Structural statistics for the ensemble of 20 lowest energy AbrBN53 dimer structures[1]

	SA_r	$\langle\text{SA}\rangle_r$	
Energy (kcal mol^{-1})			
Total	165.9 ± 3.5	158.1	
Bond lengths	3.5 ± 2	3.2	
Bond angles	126.8 ± 1.8	123.3	
Impropers	19.6 ± 0.7	18.7	
van der Waals	11.6 ± 0.8	9.7	
Restraint violations	4.6 ± 0.6	3.2	
NOE violations			
Number > 0.1 Å	0.25 ± 0.44	0	
Sum (Å)	0.03 ± 0.06	0	
Maximum (Å)	0.11	0.07	
Dihedral angles			
Number > 1.0 (°)	1.70 ± 1.56	0	
Sum of violations (°)	2.09 ± 2.02	0	
Maximum violation (°)	2.2	0.9	
R.m.s. deviations			
Intramolecular + intermolecular NOEs (Å)	0.008 ± 0002	0.006	
Dihedral angle restraints (°)	0.28 ± 008	0.154	
Ideal bond lengths (Å)	0.002 ± 00009	0.0013	
Ideal bond angles (°)	0.523 ± 001	0.495	
Ideal improper angles (°)	0.418 ± 003	0.382	
Atomic r.m.s. deviations[4] (Å)	Backbone (N,C,Cα)	2° structure	All heavy atoms
Monomer	0.30 ± 0.04	0.27 ± 0.04	0.89 ± 0.07
Dimer	0.50 ± 0.13	0.45 ± 0.11	0.99 ± 0.10

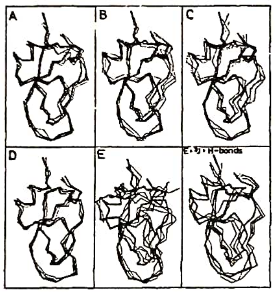
[1] X-PLOR, version 3.1 (ref. 16); distance geometry/simulated annealing and refinement <SA>, protocols were used on all structures[4,11]. <SA>, indicates the minimized average structure calculated from the 20 lowest X-PLOR energy structures.
[4] Root mean square deviation from the mean structure for residues 3–48 (backbone); 3–8, 16–29, 34–39, and 42–48 (2° structure); and 1–53 (all heavy atoms).

Root Mean Square Deviation (RMSD) – a measure of the quality of the NMR structure



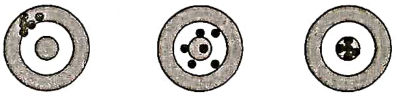


$$\mathrm{RMSD} = \sqrt{\frac{1}{N}\sum_{i=1}^{N}(r_i - r'_i)^2}$$



8 – 15 restraints/res

The number of restraints effects both precision and accuracy



Precise

Accurate

Precise and accurate